

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
6 September 2002 (06.09.2002)

PCT

(10) International Publication Number  
**WO 02/067983 A1**

- (51) International Patent Classification<sup>7</sup>: **A61K 39/145**, C12N 7/04 (74) Agent: **PRIVETT, Kathryn, Louise**; Corporate Intellectual Property, GlaxoSmithKline, CN925.1, 980 Great West Road, Brentford, Middlesex TW8 9GS (GB).
- (21) International Application Number: PCT/EP02/01843 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (22) International Filing Date: 21 February 2002 (21.02.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
0104542.6 23 February 2001 (23.02.2001) GB  
0108366.6 3 April 2001 (03.04.2001) GB
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- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 02/067983 A1

(54) Title: NOVEL VACCINE

(57) Abstract: The invention relates to the use of an influenza antigen preparation obtainable by the following process, in the manufacture of an intradermal flu vaccine: (i) harvesting of virus-containing material from a culture; (ii) clarification of the harvested material to remove non-virus material; (iii) concentration of the harvested virus; (iv) a further step to separate whole virus from non-virus material; (v) splitting of the whole virus using a suitable splitting agent for instance a detergent such as sodium deoxycholate in a density gradient centrifugation step; (vi) filtration to remove undesired materials; wherein the steps are performed in that order but not necessarily consecutively.

### Novel Vaccine

This invention relates to influenza vaccine formulations for intradermal delivery, methods for preparing them and their use in prophylaxis or therapy. More particularly  
5 the invention relates to the use of influenza vaccines which can be administered intradermally in a single dose to achieve a sufficient immune response to meet regulatory requirements.

Influenza virus is one of the most ubiquitous viruses present in the world, affecting  
10 both humans and livestock. The economic impact of influenza is significant.

The influenza virus is an RNA enveloped virus with a particle size of about 125 nm in diameter. It consists basically of an internal nucleocapsid or core of ribonucleic acid (RNA) associated with nucleoprotein, surrounded by a viral envelope with a lipid  
15 bilayer structure and external glycoproteins. The inner layer of the viral envelope is composed predominantly of matrix proteins and the outer layer mostly of the host-derived lipid material. The surface glycoproteins neuraminidase (NA) and haemagglutinin (HA) appear as spikes, 10 to 12 nm long, at the surface of the particles. It is these surface proteins, particularly the haemagglutinin, that determine  
20 the antigenic specificity of the influenza subtypes.

Typical influenza epidemics cause increases in incidence of pneumonia and lower respiratory disease as witnessed by increased rates of hospitalisation or mortality. The elderly or those with underlying chronic diseases are most likely to experience such  
25 complications, but young infants also may suffer severe disease. These groups in particular therefore need to be protected.

Currently available influenza vaccines are either inactivated or live attenuated influenza vaccines. Inactivated flu vaccines comprise one of three types of antigen  
30 preparation: inactivated whole virus, sub-virions where purified virus particles are disrupted with detergents or other reagents to solubilise the lipid envelope (so-called "split" vaccine) or purified HA and NA (subunit vaccine). These inactivated vaccines are generally given intramuscularly (i.m.).

Influenza vaccines, of all kinds, are usually trivalent vaccines. They generally contain antigens derived from two influenza A virus strains and one influenza B strain. A standard 0.5 ml injectable dose in most cases contains 15 µg of haemagglutinin antigen component from each strain, as measured by single radial immunodiffusion (SRD) (J.M. Wood et al.: An improved single radial immunodiffusion technique for the assay of influenza haemagglutinin antigen: adaptation for potency determination of inactivated whole virus and subunit vaccines. J. Biol. Stand. 5 (1977) 237-247; J. M. Wood et al., International collaborative study of single radial diffusion and immunoelectrophoresis techniques for the assay of haemagglutinin antigen of influenza virus. J. Biol. Stand. 9 (1981) 317-330).

In certain circumstances, such as the occurrence of a pandemic influenza strain, it may be desirable to have a vaccine which contains only the single strain. This will help the speed of response to a pandemic situation.

The influenza virus strains to be incorporated into influenza vaccine each season are determined by the World Health Organisation in collaboration with national health authorities and vaccine manufacturers.

Current efforts to control the morbidity and mortality associated with yearly epidemics of influenza are based on the use of intramuscularly administered inactivated influenza vaccines. The efficacy of such vaccines in preventing respiratory disease and influenza complications ranges from 75% in healthy adults to less than 50% in the elderly.

It would be desirable to provide an alternative way of administering influenza vaccines, in particular a way that is pain-free or less painful than i.m. injection, and does not involve the associated negative affect on patient compliance because of "needle fear". It would also be desirable to target the cell mediated immune system for example by targeting the antigen to the dendritic cells and langerhans cells that reside in the skin, particularly in the dermis. Cell mediated immunity appears to assist viral clearance and recovery from illness and may provide better cross protection

between influenza strains than antibodies. It has also been described in the literature that intradermal administration allows for the induction of a mucosal immunity at the level of the mucosal surfaces. This offers a benefit compared to the parenteral route for a vaccine against a pathogen such as influenza where the portal of entry of the virus is through the nasal route. Thus the mucosal surfaces, initially in the upper respiratory tract, offer the first line of defence.

Furthermore, it would be desirable to reduce the amount of antigen needed for a dose of influenza vaccine. Influenza vaccines are often in short supply.

10

Experimental intradermal exposure of humans to inactivated influenza vaccines dates back as far as the 1940s. Although the benefits of intradermal vaccination have long been recognised, there has to date been no consensus view that regular vaccination for influenza would be effective and practicable via the intradermal route.

15

Crowe (1965) Am J Medical Technology 31, 387-396 describes a study comparing intradermal and subcutaneous vaccination with a split influenza vaccine. Two doses of 0.1 ml of vaccine were administered intradermally, 14 days apart. The results obtained for intradermal delivery did not meet the standards set for two of the three strains tested, either after one or after two doses.

20

McElroy (1969) in New Eng J of Medicine, 6 November, page 1076 describes the administration of a monovalent A strain vaccine intradermally in two doses and suggests that the intradermal route might be considered when vaccine is scarce e.g. when a new, unexpected strain arises.

25

Tauraso et al (1969) Bull Wld Hlth Org 41, 507-516 describe a study using monovalent, whole inactivated influenza vaccine administered subcutaneously (0.25 ml or 0.5 ml) or intradermally (0.1 ml). A booster inoculation was given. The results suggest intradermal delivery is a reasonable alternative to subcutaneous delivery, but the authors suggest that two doses are necessary.

30

Foy (1970) in a letter to JAMA, 6/7/70, vol 213 page 130, discusses an experiment to test intradermally administered flu vaccine under natural challenge. Two doses of vaccine were given, three to four weeks apart. The data apparently suggested that intradermal vaccination did prevent disease, but were not conclusive.

5

In a letter to the British Medical Journal, 29/10/77 page 1152, an experiment using a jet gun to deliver 0.15 ml of monovalent influenza vaccine intradermally was described with unfavourable results. Intradermal administration was described as requiring further work.

10

Other authors have pointed out that intradermal injection carries with it the risk of leakage, as does subcutaneous injection. However, because of the small volume of vaccine used in intradermal administration, leakage might result in little or no protection being conferred.

15

Brooks et al (1977) Annals of Allergy 39, 110-112 describe a study in which killed influenza vaccine containing two A strains (40 CCA units of each) and separately a B strain (100 CCA units) was administered intradermally in a 0.1 ml volume. The authors concluded that the intradermal route was feasible and effective for immunisation but that larger doses than can be given intradermally may be required for certain strains.

20

Brown et al (1977) J Infectious Disease 136, 466-471 describe intradermal administration of a formalin-inactivated, whole monovalent influenza A strain vaccine. 40 CCA were used in a 0.1 ml volume. This was compared to intramuscular administration of 0.5 ml (200 CCA). The response to intradermal vaccination was found to be age-dependent and lower than for i.m. vaccination for those with preexisting antibody. The conclusion was that with the vaccination doses used in this study, intradermal vaccination should only be used in special circumstances.

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30

Halperin et al (1979) AJPH 89, 1247-1252 describe a comparison of intradermal and subcutaneous routes of influenza vaccination with a bivalent split virus vaccine. 0.1 ml of vaccine containing 40 CCA of each strain was used for the i.d. vaccination.

Herbert and Larke (1979) *J Infectious Diseases* 140, 234-238 describe a comparison of intradermal and subcutaneous influenza vaccination using a bivalent whole virus vaccine. The intradermal route was found to be less effective than the subcutaneous route where there was little or no previous exposure to the vaccine strain. The authors also observed no advantage in the smaller antigenic mass of the intradermal inoculum in relation to reactogenicity, since this did not appear to reduce side effects from the vaccine that occur with the higher dose subcutaneous immunisation.

10 Bader (1980) in a letter to *AJPH*, vol. 70 no. 5 discusses the results of various trials with intradermal delivery of flu vaccine and supports the potential value of intradermal delivery when two doses are given two weeks apart.

Niculescu et al (1981) in *Arch Roum Path Exp Microbiol*, 40, 67-70 describe intradermal administration of a split trivalent vaccine using a "gun-jet injector". Two doses were given, one month apart. The authors conclude that this method of administration can be used to decrease the rate of disease during influenza epidemics.

Thus, the literature shows an interest in intradermal vaccination between the mid-sixties (or earlier) and the early 1980s. However, the prevailing view appears to have been that two doses of vaccine would be needed. Also, there was a widely held view that due to the difficulty of administration and the lack of certainty that the low volume of vaccine would successfully be located in the desired region, the use of the intradermal delivery route would be considered only when rapid and mass vaccination was required e.g. in response to a widespread epidemic. Since the early eighties there has been little mention of intradermal flu vaccination using a protein antigen approach in the literature. Protein efforts appear to have fallen out of favour and attention was turned instead to DNA vaccination. See review by Webster R.G. (1999) in *Clin Infect Dis*, 28, 225-229 and publications such as Degano et al (1999) *Vaccine* 18, 623-32; Haensler et al (1999) *Vaccine* 17, 628-638; Degano et al (1998) *Vaccine* 16, 394-398.

Thus, the commercially available influenza vaccines remain the intramuscularly administered split or subunit injectable vaccines. These vaccines are prepared by

disrupting the virus particle, generally with an organic solvent or a detergent, and separating or purifying the viral proteins to varying extents. Split vaccines are prepared by fragmentation of whole influenza virus, either infectious or inactivated, with solubilizing concentrations of organic solvents or detergents and subsequent  
5 removal of the solubilizing agent and some or most of the viral lipid material. Split vaccines generally contain contaminating matrix protein and nucleoprotein and sometimes lipid, as well as the membrane envelope proteins. Split vaccines will usually contain most or all of the virus structural proteins although not necessarily in the same proportions as they occur in the whole virus. Subunit vaccines on the other  
10 hand consist essentially of highly purified viral surface proteins, haemagglutinin and neuraminidase, which are the surface proteins responsible for eliciting the desired virus neutralising antibodies upon vaccination. Matrix and nucleoproteins are either not detectable or barely detectable in subunit vaccines.

15 Standards are applied internationally to measure the efficacy of influenza vaccines. The European Union official criteria for an effective vaccine against influenza are set out in the table below. Theoretically, to meet the European Union requirements, an influenza vaccine has to meet only one of the criteria in the table, for all strains of influenza included in the vaccine. However in practice, at least two or more probably  
20 all three of the criteria will need to be met for all strains, particularly for a new vaccine such as a new intradermal vaccine. Under some circumstances two criteria may be sufficient. For example, it may be acceptable for two of the three criteria to be met by all strains while the third criterion is met by some but not all strains (e.g. two out of three strains). The requirements are different for adult populations (18-60  
25 years) and elderly populations (>60 years).

	18 – 60 years	> 60 years
Seroconversion rate*	>40%	>30%
Conversion factor**	>2.5	>2.0
Protection rate***	>70%	>60%

\* Seroconversion rate is defined as the percentage of vaccinees who have at least a 4-fold increase in serum haemagglutinin inhibition (HI) titres after vaccination, for each vaccine strain.

\*\* Conversion factor is defined as the fold increase in serum HI geometric mean titres (GMTs) after vaccination, for each vaccine strain.

\*\*\* Protection rate is defined as the percentage of vaccinees with a serum HI titre equal to or greater than 1:40 after vaccination (for each vaccine strain) and is normally accepted as indicating protection.

For an intradermal flu vaccine to be commercially useful it will not only need to meet those standards, but also in practice it will need to be at least as efficacious as the currently available intramuscular vaccines. It will also need to be produced by an acceptable process and will of course need to be commercially viable in terms of the amount of antigen and the number of administrations required. Furthermore, it will need to be administered using a procedure which is reliable and straightforward for medical staff to carry out.

Although intradermal flu vaccines based on inactivated virus have been studied in previous years, the fact that no intradermal flu vaccine is currently on the market reflects the difficulty to achieve effective vaccination via this route.

It has now been discovered that certain split influenza vaccines make particularly good intradermal vaccines. In particular, a single intradermal administration of such an influenza virus vaccine preparation stimulates systemic immunity at a protective level with a low dose of antigen. Furthermore, the international criteria for an effective flu vaccine are met. More specifically, intradermal administration of the low



antigen dose vaccine can produce a systemic seroconversion (4-fold increase in anti-HA titres) equivalent to that obtained by s.c. administration of the same vaccine.

As used herein, the term "intradermal delivery" means delivery of the vaccine to the region of the dermis in the skin. However, the vaccine will not necessarily be located exclusively in the dermis. The dermis is the layer in the skin located between about 1.0 and about 2.0 mm from the surface in human skin, but there is a certain amount of variation between individuals and in different parts of the body. In general, it can be expected to reach the dermis by going 1.5 mm below the surface of the skin. The dermis is located between the stratum corneum and the epidermis at the surface and the subcutaneous layer below. Depending on the mode of delivery, the vaccine may ultimately be located solely or primarily within the dermis, or it may ultimately be distributed within the epidermis and the dermis.

The invention provides in a first aspect the use of an influenza antigen preparation obtainable by the following process, in the manufacture of an intradermal flu vaccine:

- (i) harvesting of virus-containing material from a culture;
- (ii) clarification of the harvested material to remove non-virus material;
- (iii) concentration of the harvested virus;
- (iv) a further step to separate whole virus from non-virus material;
- (v) splitting of the whole virus using a suitable splitting agent in a density gradient centrifugation step;
- (vi) filtration to remove undesired materials;

wherein the steps are performed in that order but not necessarily consecutively.

Preferably the virus is grown on eggs, more particularly on embryonated hen eggs, in which case the harvested material is allantoic fluid.

Preferably the clarification step is performed by centrifugation at a moderate speed. Alternatively a filtration step may be used for example with a 0.2  $\mu\text{m}$  membrane. The clarification step gets rid of the bulk of the culture-derived e.g. egg-derived material.

Preferably the concentration step employs an adsorption method, most preferably using  $\text{CaHPO}_4$ . Alternatively filtration may be used, for example ultrafiltration.

5 Preferably the further separation step (iv) is a zonal centrifugation separation, particularly one using a sucrose gradient. Optionally the gradient contains a preservative to prevent microbial growth.

10 Preferably the splitting step is performed in a further sucrose gradient, wherein the sucrose gradient contains the splitting agent.

Preferably the filtration step (vi) is an ultrafiltration step which concentrates the split virus material.

15 Preferably there is at least one sterile filtration step, optionally at the end of the process.

Optionally there is an inactivation step prior to the final filtration step.

20 The invention provides in another aspect the use of a trivalent, split influenza antigen preparation in the manufacture of a vaccine for intradermal delivery. The split influenza antigen preparation may be produced according to the methods described herein.

25 Preferably the intradermal vaccines described herein comprise at least one non-ionic surfactant.

30 The vaccine according to the invention meets some or all of the EU criteria for influenza vaccines as set out hereinabove, such that the vaccine is approvable in Europe. Preferably, at least two out of the three EU criteria are met, for the or all strains of influenza represented in the vaccine. More preferably, at least two criteria are met for all strains and the third criterion is met by all strains or at least by all but one of the strains. Most preferably, all strains present meet all three of the criteria.

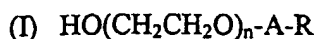
- The vaccine according to the invention has a lower quantity of haemagglutinin than conventional vaccines and is administered in a lower volume. Preferably the quantity of haemagglutinin per strain of influenza is about 1-7.5  $\mu\text{g}$  or 1-5  $\mu\text{g}$ , more preferably approximately 3  $\mu\text{g}$  or approximately 5  $\mu\text{g}$ , which is about one fifth or one third, respectively, of the dose of haemagglutinin used in conventional vaccines for intramuscular administration. 6  $\mu\text{g}$  of haemagglutinin per strain of influenza is also strongly preferred, thus 2-6.5  $\mu\text{g}$  is also a preferred range.
- 10 Preferably the volume of a dose of vaccine according to the invention is between 0.025 ml and 2.5 ml, more preferably approximately 0.1 ml or approximately 0.2 ml. A 50  $\mu\text{l}$  dose volume might also be considered. A 0.1 ml dose is approximately one fifth of the volume of a conventional intramuscular flu vaccine dose. The volume of liquid that can be administered intradermally depends in part upon the site of the injection. For example, for an injection in the deltoid region, 0.1 ml is the maximum preferred volume whereas in the lumbar region a large volume e.g. about 0.2 ml can be given.

- Preferably the vaccines according to the invention are administered to a location between about 1.0 and 2.0 mm below the surface of the skin. More preferably the vaccine is delivered to a distance of about 1.5 mm below the surface of the skin.

- The vaccine to which the invention relates is a split virion vaccine comprising particles. Preferably the vaccine contains particles having a mean particle size below 200 nm, more preferably between 50 and 180 nm, most preferably between 100 and 150 nm, as measured using a dynamic light scattering method (Malvern Zeta Sizer). Particle size may vary from season to season depending on the strains.

- The split influenza virus antigen preparation used in the present invention preferably contains at least one non-ionic surfactant. Preferably the non-ionic surfactant is at least one surfactant selected from the group consisting of the octyl- or nonylphenoxy polyoxyethanols (for example the commercially available Triton <sup>TM</sup> series),

polyoxyethylene sorbitan esters (Tween™ series) and polyoxyethylene ethers or esters of general formula (I):



wherein n is 1-50, A is a bond or -C(O)-, R is C<sub>1-50</sub> alkyl or phenyl C<sub>1-50</sub> alkyl; and  
5 combinations of two or more of these.

Preferred surfactants falling within formula (I) are molecules in which n is 4-24, more preferably 6-12, and most preferably 9; the R component is C<sub>1-50</sub>, preferably C<sub>4</sub>-C<sub>20</sub> alkyl and most preferably C<sub>12</sub> alkyl.

10

Octylphenoxy polyoxyethanols and polyoxyethylene sorbitan esters are described in "Surfactant systems" Eds: Attwood and Florence (1983, Chapman and Hall).

Octylphenoxy polyoxyethanols (the octoxynols), including t-octylphenoxy polyethoxyethanol (Triton X-100™) are also described in Merck Index  
15 Entry 6858 (Page 1162, 12<sup>th</sup> Edition, Merck & Co. Inc., Whitehouse Station, N.J., USA; ISBN 0911910-12-3). The polyoxyethylene sorbitan esters, including polyoxyethylene sorbitan monooleate (Tween 80™) are described in Merck Index Entry 7742 (Page 1308, 12<sup>th</sup> Edition, Merck & Co. Inc., Whitehouse Station, N.J., USA; ISBN 0911910-12-3). Both may be manufactured using methods described  
20 therein, or purchased from commercial sources such as Sigma Inc.

Particularly preferred non-ionic surfactants include Triton X-45, t-octylphenoxy polyethoxyethanol (Triton X-100), Triton X-102, Triton X-114, Triton X-165, Triton X-205, Triton X-305, Triton N-57, Triton N-101, Triton N-128, Breij 35,  
25 polyoxyethylene-9-lauryl ether (laureth 9) and polyoxyethylene-9-stearyl ether (steareth 9). Triton X-100 and laureth 9 are particularly preferred. Also particularly preferred is the polyoxyethylene sorbitan ester, polyoxyethylene sorbitan monooleate (Tween 80™).

30 Further suitable polyoxyethylene ethers of general formula (I) are selected from the following group: polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

Alternative terms or names for polyoxyethylene lauryl ether are disclosed in the CAS registry. The CAS registry number of polyoxyethylene-9 lauryl ether is: 9002-92-0.

Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12<sup>th</sup> ed: entry 7717, Merck & Co. Inc., Whitehouse Station, N.J., USA; 5 ISBN 0911910-12-3). Laureth 9 is formed by reacting ethylene oxide with dodecyl alcohol, and has an average of nine ethylene oxide units.

The ratio of the length of the polyoxyethylene section to the length of the alkyl chain in the surfactant (*i.e.* the ratio of  $n$ : alkyl chain length), affects the solubility of this 10 class of surfactant in an aqueous medium. Thus, the surfactants of the present invention may be in solution or may form particulate structures such as micelles or vesicles. As a solution, the surfactants of the present invention are safe, easily sterilisable, simple to administer, and may be manufactured in a simple fashion without the GMP and QC issues associated with the formation of uniform particulate 15 structures. Some polyoxyethylene ethers, such as laureth 9, are capable of forming non-vesicular solutions. However, polyoxyethylene-8 palmitoyl ether ( $C_{18}E_8$ ) is capable of forming vesicles. Accordingly, vesicles of polyoxyethylene-8 palmitoyl ether in combination with at least one additional non-ionic surfactant, can be employed in the formulations of the present invention.

20 Preferably, the polyoxyethylene ether used in the formulations of the present invention has haemolytic activity. The haemolytic activity of a polyoxyethylene ether may be measured *in vitro*, with reference to the following assay, and is as expressed as the highest concentration of the surfactant which fails to cause lysis of the red blood cells:

25

1. Fresh blood from guinea pigs is washed with phosphate buffered saline (PBS) 3 times in a desk-top centrifuge. After re-suspension to the original volume the blood is further diluted 10 fold in PBS.
2. 50  $\mu$ l of this blood suspension is added to 800  $\mu$ l of PBS containing two-fold 30 dilutions of detergent.
3. After 8 hours the haemolysis is assessed visually or by measuring the optical density of the supernatant. The presence of a red supernatant, which absorbs light at 570 nm indicates the presence of haemolysis.

4. The results are expressed as the concentration of the first detergent dilution at which hemolysis no longer occurs.

Within the inherent experimental variability of such a biological assay, the  
5 polyoxyethylene ethers, or surfactants of general formula (I), of the present invention preferably have a haemolytic activity, of approximately between 0.5-0.0001%, more preferably between 0.05-0.0001%, even more preferably between 0.005-0.0001%, and most preferably between 0.003-0.0004%. Ideally, said polyoxyethylene ethers or esters should have a haemolytic activity similar (*i.e.* within a ten-fold difference) to  
10 that of either polyoxyethylene-9 lauryl ether or polyoxyethylene-8 stearyl ether.

Two or more non-ionic surfactants from the different groups of surfactants described may be present in the vaccine formulation described herein. In particular, a combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan  
15 monooleate (Tween 80™) and an octoxynol such as t-octylphenoxypolyethoxyethanol (Triton) X-100™ is preferred. Another particularly preferred combination of non-ionic surfactants comprises laureth 9 plus a polyoxyethylene sorbitan ester or an octoxynol or both.

20 Preferably the or each non-ionic surfactant is present in the final vaccine formulation at a concentration of between 0.001 to 20%, more preferably 0.01 to 10%, and most preferably up to about 2% (w/v). Where one or two surfactants are present, these are generally present in the final formulation at a concentration of up to about 2% each, typically at a concentration of up to about 0.6% each. One or more additional  
25 surfactants may be present, generally up to a concentration of about 1% each and typically in traces up to about 0.2% or 0.1 % each. Any mixture of surfactants may be present in the vaccine formulations according to the invention.

Non-ionic surfactants such as those discussed above have preferred concentrations in  
30 the final vaccine composition as follows: polyoxyethylene sorbitan esters such as Tween 80™: 0.01 to 1%, most preferably about 0.1% (w/v); octyl- or nonylphenoxy polyoxyethanols such as Triton X-100™ or other detergents in the Triton series: 0.001 to 0.1%, most preferably 0.005 to 0.02 % (w/v); polyoxyethylene ethers of general

formula (I) such as laureth 9: 0.1 to 20 %, preferably 0.1 to 10 % and most preferably 0.1 to 1 % or about 0.5% (w/v).

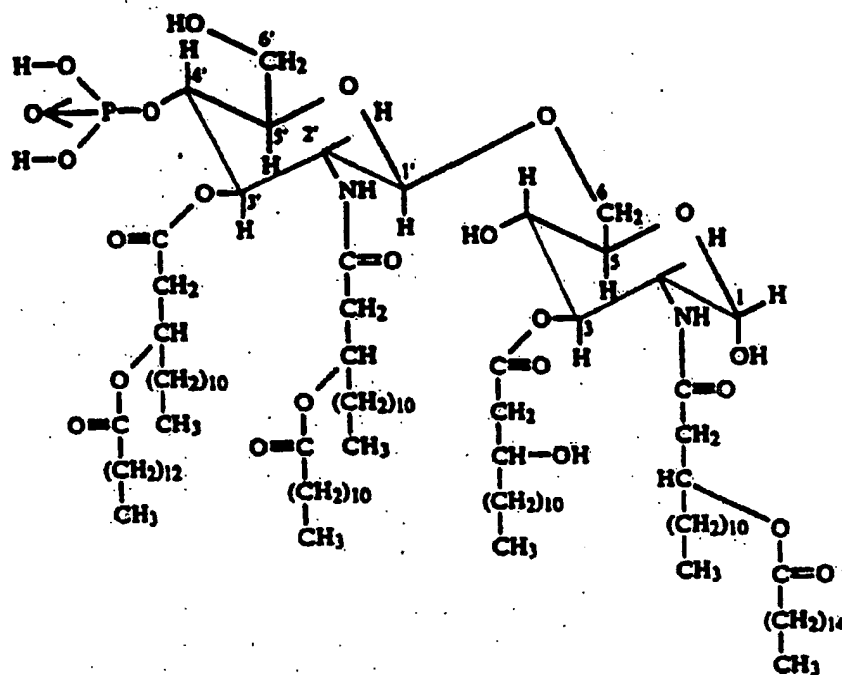
Other reagents may also be present in the formulation. As such the formulations of  
5 the present invention may also comprise a bile acid or a derivative thereof, in particular in the form of a salt. These include derivatives of cholic acid and salts thereof, in particular sodium salts of cholic acid or cholic acid derivatives. Examples of bile acids and derivatives thereof include cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid, ursodeoxycholic acid, hyodeoxycholic acid  
10 and derivatives such as glyco-, tauro-, amidopropyl-1-propanesulfonic-, amidopropyl-2-hydroxy-1-propanesulfonic derivatives of the aforementioned bile acids, or N,N-bis(3Dgluconoamidopropyl) deoxycholamide. A particularly preferred example is sodium deoxycholate (NaDOC) which may be present in the final vaccine dose.

15 The vaccine formulation according to the invention preferably comprises a split flu virus preparation in combination with one or more non-ionic surfactants. The one or more non-ionic surfactants may be residual from the process by which the split flu antigen preparation is produced, and/or added to the antigen preparation later. The concentration of the or each non-ionic surfactant may be adjusted to the desired level  
20 at the end of the splitting/purification process. It is believed that the split flu antigen material may be stabilised in the presence of a non-ionic surfactant, though it will be understood that the invention does not depend upon this necessarily being the case.

The vaccine according to the invention may further comprise an adjuvant or  
25 immunostimulant such as but not limited to detoxified lipid A from any source and non-toxic derivatives of lipid A, saponins and other reagents capable of stimulating a TH1 type response.

It has long been known that enterobacterial lipopolysaccharide (LPS) is a potent  
30 stimulator of the immune system, although its use in adjuvants has been curtailed by its toxic effects. A non-toxic derivative of LPS, monophosphoryl lipid A (MPL), produced by removal of the core carbohydrate group and the phosphate from the reducing-end glucosamine, has been described by Ribí et al (1986, Immunology and

Immunopharmacology of bacterial endotoxins, Plenum Publ. Corp., NY, p407-419)  
and has the following structure:



- A further detoxified version of MPL results from the removal of the acyl chain from the 3-position of the disaccharide backbone, and is called 3-O-Deacylated monophosphoryl lipid A (3D-MPL). It can be purified and prepared by the methods taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof.
- 10 A preferred form of 3D-MPL is in the form of an emulsion having a small particle size less than 0.2µm in diameter, and its method of manufacture is disclosed in WO 94/21292. Aqueous formulations comprising monophosphoryl lipid A and a surfactant have been described in WO9843670A2.
- 15 The bacterial lipopolysaccharide derived adjuvants to be formulated in the compositions of the present invention may be purified and processed from bacterial sources, or alternatively they may be synthetic. For example, purified monophosphoryl lipid A is described in Ribí et al 1986 (supra), and 3-O-Deacylated monophosphoryl or diphosphoryl lipid A derived from *Salmonella sp.* is described in GB 2220211 and
- 20 US 4912094. Other purified and synthetic lipopolysaccharides have been described



(Hilgers *et al.*, 1986, *Int.Arch.Allergy.Immunol.*, 79(4):392-6; Hilgers *et al.*, 1987, *Immunology*, 60(1):141-6; and EP 0 549 074 B1). A particularly preferred bacterial lipopolysaccharide adjuvant is 3D-MPL.

- 5 Accordingly, the LPS derivatives that may be used in the present invention are those immunostimulants that are similar in structure to that of LPS or MPL or 3D-MPL. In another aspect of the present invention the LPS derivatives may be an acylated monosaccharide, which is a sub-portion to the above structure of MPL.
- 10 Saponins are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. *Phytomedicine* vol 2 pp 363-386). Saponins are steroid or triterpene glycosides widely distributed in the plant and marine animal kingdoms. Saponins are noted for forming colloidal solutions in water which foam on shaking, and for precipitating cholesterol. When saponins are near cell
- 15 membranes they create pore-like structures in the membrane which cause the membrane to burst. Haemolysis of erythrocytes is an example of this phenomenon, which is a property of certain, but not all, saponins.

- Saponins are known as adjuvants in vaccines for systemic administration. The
- 20 adjuvant and haemolytic activity of individual saponins has been extensively studied in the art (Lacaille-Dubois and Wagner, *supra*). For example, Quil A (derived from the bark of the South American tree *Quillaja Saponaria* Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1.
- 25 Particulate structures, termed Immune Stimulating Complexes (ISCOMS), comprising fractions of Quil A are haemolytic and have been used in the manufacture of vaccines (Morein, B., EP 0 109 942 B1; WO 96/11711; WO 96/33739). The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US
- 30 Patent No.5,057,540 and EP 0 362 279 B1. Other saponins which have been used in systemic vaccination studies include those derived from other plant species such as *Gypsophila* and *Saponaria* (Bomford *et al.*, *Vaccine*, 10(9):572-577, 1992).

An enhanced system involves the combination of a non-toxic lipid A derivative and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739.

5

A particularly potent adjuvant formulation involving QS21 and 3D-MPL in an oil in water emulsion is described in WO 95/17210 and is a preferred formulation.

Accordingly in one embodiment of the present invention there is provided a vaccine  
10 comprising an influenza antigen preparation of the present invention adjuvanted with detoxified lipid A or a non-toxic derivative of lipid A, more preferably adjuvanted with a monophosphoryl lipid A or derivative thereof.

Preferably the vaccine additionally comprises a saponin, more preferably QS21.

15

Preferably the formulation additionally comprises an oil in water emulsion. The present invention also provides a method for producing a vaccine formulation comprising mixing an antigen preparation of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

20

Additional components that are preferably present in an adjuvanted vaccine formulation according to the invention include non-ionic detergents such as the octoxynols and polyoxyethylene esters as described herein, particularly t-octylphenoxy polyethoxyethanol (Triton X-100) and polyoxyethylene sorbitan monooleate (Tween  
25 80); and bile salts or cholic acid derivatives as described herein, in particular sodium deoxycholate or taurodeoxycholate. Thus, a particularly preferred formulation comprises 3D-MPL, Triton X-100, Tween 80 and sodium deoxycholate, which may be combined with an influenza virus antigen preparation to provide a vaccine suitable for intradermal application.

30

In one preferred embodiment of the present invention, the intradermal influenza vaccines comprise a vesicular adjuvant formulation comprising cholesterol, a saponin and an LPS derivative. In this regard the preferred adjuvant formulation comprises a

unilamellar vesicle comprising cholesterol, having a lipid bilayer preferably comprising dioleoyl phosphatidyl choline, wherein the saponin and the LPS derivative are associated with, or embedded within, the lipid bilayer. More preferably, these adjuvant formulations comprise QS21 as the saponin, and 3D-MPL as the LPS derivative, wherein the ratio of QS21:cholesterol is from 1:1 to 1:100 weight/weight, and most preferably 1:5 weight/weight. Such adjuvant formulations are described in EP 0 822 831 B, the disclosure of which is incorporated herein by reference.

The invention also provides a method for the prophylaxis of influenza infection or disease in a subject which method comprises administering to the subject intradermally a split influenza vaccine according to the invention.

The invention provides in a further aspect a pharmaceutical kit comprising an intradermal administration device and a vaccine formulation as described herein. The device is preferably supplied already filled with the vaccine. Preferably the vaccine is in a liquid volume smaller than for conventional intramuscular vaccines as described herein, particularly a volume of between about 0.05 ml and 0.2 ml. Preferably the device is a short needle delivery device for administering the vaccine to the dermis.

Suitable devices for use with the intradermal vaccines described herein include short needle devices such as those described in US 4,886,499, US 5,190,521, US 5,328,483, US 5,527,288, US 4,270,537, US 5,015,235, US 5,141,496, US 5,417,662.

Intradermal vaccines may also be administered by devices which limit the effective penetration length of a needle into the skin, such as those described in WO99/34850, incorporated herein by reference, and functional equivalents thereof. Also suitable are jet injection devices which deliver liquid vaccines to the dermis via a liquid jet injector or via a needle which pierces the stratum corneum and produces a jet which reaches the dermis. Jet injection devices are described for example in US 5,480,381, US 5,599,302, US 5,334,144, US 5,993,412, US 5,649,912, US 5,569,189, US 5,704,911, US 5,383,851, US 5,893,397, US 5,466,220, US 5,339,163, US 5,312,335, US 5,503,627, US 5,064,413, US 5,520, 639, US 4,596,556, US 4,790,824, US 4,941,880, US 4,940,460, WO 97/37705 and WO 97/13537. Also suitable are ballistic powder/particle delivery devices which use compressed gas to accelerate

vaccine in powder form through the outer layers of the skin to the dermis. Additionally, conventional syringes may be used in the classical mantoux method of intradermal administration. However, the use of conventional syringes requires highly skilled operators and thus devices which are capable of accurate delivery without a highly skilled user are preferred.

The influenza vaccine according to the invention is preferably a multivalent influenza vaccine comprising two or more strains of influenza. Most preferably it is a trivalent vaccine comprising three strains. Conventional influenza vaccines comprise three strains of influenza, two A strains and one B strain. However, monovalent vaccines, which may be useful for example in a pandemic situation, are not excluded from the invention. A monovalent, pandemic flu vaccine will most likely contain influenza antigen from a single A strain.

The influenza virus preparations may be derived from the conventional embryonated egg method, or they may be derived from any of the new generation methods using tissue culture to grow the virus. Suitable cell substrates for growing the virus include for example dog kidney cells such as MDCK or cells from a clone of MDCK, MDCK-like cells, monkey kidney cells such as AGMK cells including Vero cells, or any other mammalian cell type suitable for the production of influenza virus for vaccine purposes. Suitable cell substrates also include human cells e.g. MRC-5 cells. Suitable cell substrates are not limited to cell lines; for example primary cells such as chicken embryo fibroblasts are also included.

Traditionally split flu was produced using a solvent/detergent treatment, such as tri-*n*-butyl phosphate, or diethylether in combination with Tween<sup>TM</sup> (known as "Tween-ether" splitting) and this process is still used in some production facilities. Other splitting agents now employed include detergents or proteolytic enzymes or bile salts, for example sodium deoxycholate as described in patent no. DD 155 875, incorporated herein by reference. Detergents that can be used as splitting agents include cationic detergents e.g. cetyl trimethyl ammonium bromide (CTAB), other ionic detergents e.g. laurylsulfate, taurodeoxycholate, or non-ionic detergents such as the ones described above including Triton X-100 (for example in a process described

in Lina et al, 2000, Biologicals 28, 95-103) and Triton N-101, or combinations of any two or more detergents.

Further suitable splitting agents which can be used to produce split flu virus  
5 preparations include:

1. Bile acids and derivatives thereof including: cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid, ursodeoxycholic acid, hyodeoxycholic acid and derivatives like glyco-, tauro-, amidopropyl-1-propanesulfonic-, amidopropyl-2-  
10 hydroxy-1-propanesulfonic derivatives of the aforementioned bile acids, or N,N-bis (3D-Gluconoamidopropyl) deoxycholamide. A particular example is sodium deoxycholate (NaDOC) which may be present in trace amounts in the final vaccine dose.
- 15 2. Alkylglycosides or alkylthioglycosides, where the alkyl chain is between C6 - C18, typical between C8 and C14, sugar moiety is any pentose or hexose or combinations thereof with different linkages, like 1->6, 1->5, 1->4, 1->3, 1-2. The alkyl chain can be saturated, unsaturated and/or branched.
- 20 3. Derivatives of 2 above, where one or more hydroxyl groups, preferably the 6 hydroxyl group is/are modified, like esters, ethoxylates, sulphates, ethers, carbonates, sulphasuccinates, isethionates, ethercarboxylates, quaternary ammonium compounds.
4. Acyl sugars, where the acyl chain is between C6 and C18, typical between C8  
25 and C12, sugar moiety is any pentose or hexose or combinations thereof with different linkages, like 1->6, 1->5, 1->4, 1->3, 1-2. The acyl chain can be saturated or unsaturated and/or branched, cyclic or non-cyclic, with or without one or more heteroatoms e.g. N, S, P or O.
- 30 5. Sulphobetaines of the structure R-N,N-(R1,R2)-3-amino-1-propanesulfonate, where R is any alkyl chain or arylalkyl chain between C6 and C18, typical between C8 and C16. The alkyl chain R can be saturated, unsaturated and/or branched. R1 and R2

are preferably alkyl chains between C1 and C4, typically C1, or R1, R2 can form a heterocyclic ring together with the nitrogen.

6. Betains of the structure R-N,N-(R1,R2)-glycine, where R is any alkyl chain  
5 between C6 and C18, typical between C8 and C16. The alkyl chain can be saturated  
unsaturated and/or branched. R1 and R2 are preferably alkyl chains between C1 and  
C4, typically C1, or R1 and R2 can form a heterocyclic ring together with the  
nitrogen.
- 10 7. N,N-dialkyl-glucamides, of the Structure R-(N-R1)-glucamide, where R is any  
alkylchain between C6 and C18, typical between C8 and C12. The alkyl chain can be  
saturated unsaturated and/or branched or cyclic. R1 and R2 are alkyl chains between  
C1 and C6, typically C1. The sugar moiety might be modified with pentoses or  
hexoses.
- 15 8. Quarternary ammonium compounds of the structure R, -N<sup>+</sup> (-R1, -R2, -R3),  
where R is any alkylchain between C6 and C20, typically C20. The alkyl chain can be  
saturated unsaturated and/or branched. R1, R2 and R3 are preferably alkyl chains  
between C1 and C4, typically C1, or R1, R2 can form a heterocyclic ring together with  
20 the nitrogen. A particular example is cetyl trimethyl ammonium bromide (CTAB).

The preparation process for a split vaccine will include a number of different filtration  
and/or other separation steps such as ultracentrifugation, ultrafiltration, zonal  
centrifugation and chromatography (e.g. ion exchange) steps in a variety of  
25 combinations, and optionally an inactivation step eg with formaldehyde or  $\beta$ -  
propiolactone or U.V. which may be carried out before or after splitting. The splitting  
process may be carried out as a batch, continuous or semi-continuous process.

Preferably, a bile salt such as sodium deoxycholate is present in trace amounts in a  
30 split vaccine formulation according to the invention, preferably at a concentration not  
greater than 0.05%, or not greater than about 0.01%, more preferably at about  
0.0045% (w/v).

Preferred split flu vaccine antigen preparations according to the invention comprise a residual amount of Tween 80 and/or Triton X-100 remaining from the production process, although these may be added or their concentrations adjusted after preparation of the split antigen. Preferably both Tween 80 and Triton X-100 are present. The preferred ranges for the final concentrations of these non-ionic surfactants in the vaccine dose are:

Tween 80: 0.01 to 1%, more preferably about 0.1% (v/v)

Triton X-100: 0.001 to 0.1 (% w/v), more preferably 0.005 to 0.02% (w/v).

The presence of the combination of these two surfactants, in low concentrations, was found to promote the stability of the antigen in solution. It is possible that this enhanced stability rendered the antigen more immunogenic intradermally than previous formulations have been. Such an enhancement could arise from a prevalence of small antigen aggregates or the enhancement of the native conformation of the antigen. It will be appreciated that the invention does not depend upon this theoretical explanation being correct.

In a particular embodiment, the preferred split virus preparation also contains laureth 9, preferably in the range 0.1 to 20%, more preferably 0.1 to 10% and most preferably 0.1 to 1% (w/v).

The vaccines according to the invention generally contain not more than 25% (w/v) of detergent or surfactant, preferably less than 15% and most preferably not more than about 2%.

The invention will now be further described in the following, non-limiting examples.

## EXAMPLES

### Example 1 – Preparation of split influenza vaccine

- 5 Each strain for the split vaccine was prepared according to the following procedure.

#### **Preparation of virus inoculum**

- On the day of inoculation of embryonated eggs a fresh inoculum is prepared by mixing the working seed lot with a phosphate buffered saline containing gentamycin sulphate at 0.5 mg/ml and hydrocortisone at 25 µg/ml. (virus strain-dependent). The virus inoculum is kept at 2-8°C.
- 10

#### **Inoculation of embryonated eggs**

- Nine to eleven day old embryonated eggs are used for virus replication. Shells are decontaminated. The eggs are inoculated with 0.2 ml of the virus inoculum. The inoculated eggs are incubated at the appropriate temperature (virus strain-dependent) for 48 to 96 hours. At the end of the incubation period, the embryos are killed by cooling and the eggs are stored for 12-60 hours at 2-8°C.
- 15

#### **20 Harvest**

The allantoic fluid from the chilled embryonated eggs is harvested. Usually, 8 to 10 ml of crude allantoic fluid is collected per egg. To the crude monovalent virus bulk 0.100 mg/ml thiomersal is optionally added.

#### **25 Concentration and purification of whole virus from allantoic fluid**

##### **1. Clarification**

The harvested allantoic fluid is clarified by moderate speed centrifugation (range: 4000 – 14000 g).

##### **30 2. Adsorption step**

To obtain a CaHPO<sub>4</sub> gel in the clarified virus pool, 0.5 mol/L Na<sub>2</sub>HPO<sub>4</sub> and 0.5mol/L CaCl<sub>2</sub> solutions are added to reach a final concentration of CaHPO<sub>4</sub> of 1.5 g to 3.5 g CaHPO<sub>4</sub>/litre depending on the virus strain.



After sedimentation for at last 8 hours, the supernatant is removed and the sediment containing the influenza virus is resolubilised by addition of a 0.26 mol/L EDTA-Na<sub>2</sub> solution, dependent on the amount of CaHPO<sub>4</sub> used.

5

### 3. Filtration

The resuspended sediment is filtered on a 6µm filter membrane.

### 4. Sucrose gradient centrifugation

- 10 The influenza virus is concentrated by isopycnic centrifugation in a linear sucrose gradient (0 - 55 % (w/v)) containing 100 µg/ml Thiomersal. The flow rate is 8 – 15 litres/hour.

- At the end of the centrifugation, the content of the rotor is recovered by four different  
15 fractions (the sucrose is measured in a refractometer):

- fraction 1 55-52% sucrose
- fraction 2 approximately 52-38% sucrose
- fraction 3 38-20% sucrose\*
- fraction 4 20- 0% sucrose

- 20 \* virus strain-dependent: fraction 3 can be reduced to 15% sucrose.

For further vaccine preparation, only fractions 2 and 3 are used.

- 25 Fraction 3 is washed by diafiltration with phosphate buffer in order to reduce the sucrose content to approximately below 6%. The influenza virus present in this diluted fraction is pelleted to remove soluble contaminants.

- The pellet is resuspended and thoroughly mixed to obtain a homogeneous suspension. Fraction 2 and the resuspended pellet of fraction 3 are pooled and phosphate buffer is  
30 added to obtain a volume of approximately 40 litres. This product is the monovalent whole virus concentrate.

### 5. Sucrose gradient centrifugation with sodium deoxycholate

The monovalent whole influenza virus concentrate is applied to a ENI-Mark II ultracentrifuge. The K3 rotor contains a linear sucrose gradient (0 - 55 % (w/v)) where a sodium deoxycholate gradient is additionally overlayed. Tween 80 is present during splitting up to 0.1 % (w/v). The maximal sodium deoxycholate concentration is 0.7-  
5 1.5 % (w/v) and is strain dependent. The flow rate is 8 – 15 litres/hour.

At the end of the centrifugation, the content of the rotor is recovered by three different fractions (the sucrose is measured in a refractometer) Fraction 2 is used for further processing. Sucrose content for fraction limits (47-18%) varies according to strains  
10 and is fixed after evaluation:

#### 6. Sterile filtration

The split virus fraction is filtered on filter membranes ending with a 0.2 µm membrane. Phosphate buffer containing 0.025 % (w/v) Tween 80 is used for dilution.  
15 The final volume of the filtered fraction 2 is 5 times the original fraction volume.

#### 7. Inactivation

The filtered monovalent material is incubated at  $22 \pm 2^{\circ}\text{C}$  for at most 84 hours (dependent on the virus strains, this incubation can be shortened). Phosphate buffer  
20 containing 0.025% Tween 80 is then added in order to reduce the total protein content down to max. 250 µg/ml. Formaldehyde is added to a final concentration of 50 µg/ml and the inactivation takes place at  $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for at least 72 hours.

#### 8. Ultrafiltration

25 The inactivated split virus material is concentrated at least 2 fold in a ultrafiltration unit, equipped with cellulose acetate membranes with 20 kDa MWCO. The Material is subsequently washed with phosphate buffer containing 0.025 % (w/v) Tween 80 and following with phosphate buffered saline containing 0.01 % (w/v) Tween.

#### 30 9. Final sterile filtration

The material after ultrafiltration is filtered on filter membranes ending with a 0.2 µm membrane. The final concentration of Haemagglutinin, measured by SRD (method recommended by WHO) should exceed 450 µg/ml.

## 5 10. Storage

The monovalent final bulk is stored at 2 – 8°C for a maximum of 18 months.

### Purity

Purity was determined semiquantitatively by O.D. scanning of Coomassie-stained  
10 polyacrylamide gels. Peaks were determined manually. Sample results are given in Table 1.

**Table 1**

Viral Proteins (HA, NP, M) %					Other viral and host-cell derived proteins %
H3N2	HA dimer	HA1 + 2	NP	M	
A/Syd/5/97	10.34	22.34	25.16	37.33	4.83
A/Nan933/95	8.17	15.8	40.09	30.62	5.32
<b>B</b>					
B/Har/7/94	5.71 <sup>2</sup>	24.07	15.64	50	4.58
B/Yam/166/98	0.68	27.62	21.48	46.02	4.2
<b>H1N1</b>					
A/Tex/36/91		33.42	24.46	34.33	7.79
A/Bei/262/95		32.73	35.72	27.06	4.49
<b>H2N2</b>					
A/sing/1/57	2.8	39.7	21.78	32.12	3.6

15 A particular combination of strains for use in the invention includes A/New Caledonia/20/99 (H1N1), A/Panama/20/99 (H3N2) and B/Yamanashi/166/98.

**Example 2 – Preparation of vaccine doses from bulk vaccine**

- 5 Final vaccine is prepared by formulating a trivalent vaccine from the monovalent bulks with the detergent concentrations adjusted as required.

PBS, pH 7.2+/-0.2, Tween 80 and Triton X-100 are mixed to obtain the required final concentrations (PBS 1x concentrated, Tween 80 0.15% and Triton X-100 0.02%) .

- 10 The three following inactivated split virions are added with 10 minutes stirring in between:

15µg A/New Caledonia/20/99 (H1N1)

15µg A/Panama/20/99 (H3N2)

15µg B/Yamanashi/166/98

15

After 15 minutes stirring pH is adjusted to 7.2+/-0.2.

The dose volume is 500µl. The doses are filled in sterile ampoules. Immediately before applying the vaccine, 0.1 ml doses are removed from the ampoule using the device for intradermal application.

20

**Example 3 – Methods used to measure antibody responses****1. Detection of specific anti-Flu and total IgA in human nasal secretions by ELISA**

- 25 *Collection method for human nasal secretions*

An appropriate method is used to collect nasal secretions, for example a classical nasal wash method or a nasal wick method.

- 30 After collection and treatment of human nasal secretions, the detection of total and specific anti-FLU IgA is realized with ELISAs e.g:

*Capture ELISA for detection of total IgA*

Total IgA are captured with anti-human IgA polyclonal affinity purified Ig immobilized on microtiter plates and subsequently detected using a different polyclonal anti-human IgA affinity purified Ig coupled to peroxidase.

- 5 A purified human sIgA is used as a standard to allow the quantification of sIgA in the collected nasal secretions.

3 references of purified human sIgA are used as low, medium and high references in this assay.

10

*Direct ELISA for detection of specific anti-FLU IgA*

Three different ELISAs are performed, one on each FLU strain present in the vaccine formulation.

- 15 Specific anti-FLU IgA are captured with split inactivated FLU antigens coated on microtiter plates and subsequently detected using the same different polyclonal anti-human IgA affinity purified Ig coupled to peroxidase as the one used for the total IgA ELISA.

20 **Results - expression and calculations**

*Total IgA expression*

The results are expressed as  $\mu\text{g}$  of total IgA in 1 ml of nasal fluids, using a Softmaxpro program.

*Specific anti-Flu IgA expression*

- 25 The results are expressed as end-point unit titer, which are calculated as the inverse of the last dilution which gives an  $\text{OD}_{450\text{nm}}$  above the cut off .

The final results of a sample are expressed as follows:

Normalization of the specific response by calculating the ratio between the specific response and the total IgA concentration: end-point unit/ $\mu\text{g}$  total IgA ( most

- 30 commonly used calculation method in the literature).

## 2. Haemagglutination Inhibition (HAI) activity of Flu-specific serum Abs

Sera (50 µl) are treated with 200 µl RDE (receptor destroying enzyme) for 16 hours at 37°C. The reaction is stopped with 150 µl 2.5% Na citrate and the sera are inactivated at 56°C for 30 min. A dilution 1:10 is prepared by adding 100 µl PBS. Then, a 2-fold dilution series is prepared in 96 well plates (V-bottom) by diluting 25 µl serum (1:10) with 25 µl PBS. 25 µl of the reference antigens are added to each well at a concentration of 4 hemagglutinating units per 25 µl. Antigen and antiserum dilution are mixed using a microtiter plate shaker and incubated for 60 minutes at room temperature. 50 µl chicken red blood cells (RBC) (0.5%) are then added and the RBCs are allowed to sediment for 1 hour at RT. The HAI titre corresponds to the inverse of the last serum dilution that completely inhibits the virus-induced hemagglutination.

15

### Example 4 – Immunogenicity and Reactogenicity of Flu ID

Clinical trials were carried out on human subjects to assess efficacy of the influenza vaccine of the invention delivered ID. The vaccine (Fluarix™) used in this study was made according to Examples 1 and 2.

A hundred healthy male and female volunteers (18-60 years of age) were enrolled and randomised in 2 groups (50 subjects per group). The vaccine was administered according two route of administration.

25

- Intramuscularly administered trivalent split influenza vaccine (Fluarix™):  
1 dose → Day 0.

The vaccine was supplied as a pre-filled syringe for intramuscular injection in the deltoid region of the non-predominant arm. In order to ensure proper intramuscular injection of the study vaccines, a needle of at least 23G (2.2 cm/1in.) length was used.

30

- Intradermally administered trivalent split influenza vaccine (Fluarix™):

1/5 dose → Day 0

5 The vaccine was supplied as 0.5ml ampoule dose. 1/5 of the full dose (100µl) was injected intradermally using a device as disclosed in EP1092444, the whole contents of which are herein incorporated by reference. The device has a skin contacting element that effectively limits the penetration depth of the needle into the dermis. Effective needle length was approximately 1.5 mm. This device is herein referred to as the ID delivery device or 'IDD'.

10 The duration of the study was approximately 21 days per subjects with only one dose of the vaccine given intramuscularly or intradermally according to the group. Blood was sampled at day 0 and 21.

The study population were as follows:

Group 1	Group 2
Fluarix™ Intramuscular	Fluarix™ Intradermal with IDD
0.5 ml of Fluarix™, lot n° 18500A9	0.1 ml of Fluarix™, lot n°18526B7
N= 50	N= 50

15

The demographic profile of the 2 groups of subjects who received vaccine was comparable with respect to mean age, gender and racial distribution.

### Immunogenicity

For each treatment group, the following parameters for immunogenicity were  
20 calculated:

- Geometric mean titres (GMTs) (with 95% confidence intervals) of HI antibody titres at days 0 and 21, calculated by taking the anti-log of the mean of the log titre transformations (titres below the cut-off value were given the arbitrary value of  
25 half the cut-off for calculation purpose).

- Seropositivity (S+) rates of HI antibody titres at days 0 and 21, defined as the percentage of subjects with titre greater than or equal to the assay cut-off.
- Conversion factors at day 21 defined as the fold increase in serum HI GMTs on day 21 compared to day 0
- 5 • Seroconversion rates (SC) at day 21 defined as the percentage of vaccinees who have at least a 4-fold increase in serum HI titres on day 21 compared to day 0
- Protection rates at day 21 defined as the percentage of vaccinees with a serum HI titre  $\geq 1:40$  after vaccination.

#### 10 Laboratory assays and timepoints

All serum samples were kept at  $-20^{\circ}\text{C}$  and adequate measures taken to ensure that samples did not thaw at any time. At each visit, blood was collected for measurement of HI antibody response.

- 15 The immune response was determined by the titre of haemagglutination-inhibiting antibodies (HAI) measured by the haemagglutination-inhibition test described by the WHO Collaborating Centre for Influenza, Centres for Diseases Control, Atlanta, USA (1991).

- 20 Frozen serum samples were received at Sächsisches Serumwerk GmbH (SSW), Dresden, Germany and antibody determination was conducted on samples after thawing, with a standardised and comprehensively validated micromethod using 4 haemagglutination-inhibiting units (4 HIU) of the appropriate antigens and a 0.5% fowl erythrocyte suspension. The antigens A (H3N2 and H1N1) were obtained as
- 25 whole virus antigens from the allantoic fluid of embryonated hens' eggs. The B antigen was subjected to cleavage with a mixture of ether and Tween 80 to increase sensitivity. Non-specific serum inhibitors were removed by heat treatment and receptor-destroying enzyme.

- 30 The sera obtained are evaluated for HI antibody levels. Starting with an initial dilution of 1:10, a dilution series (by a factor of 2) is prepared up to an end dilution of



1:20480. The titration end-point is taken as the highest dilution step that shows complete inhibition (100%) of haemagglutination. All assays are performed in duplicate.

## 5 Results

The number of subjects being the same in the ATP immunogenicity cohort and the total cohort, the immunogenicity analysis was performed only on an intent-to-treat (ITT) basis (i.e. total cohort).

### HI titres and conversion factors

- 10 The Geometric mean titres (GMTs) (with 95% confidence intervals) of HI antibody titres at days 0 and 21, for the three groups are given in the table below:

#### *Seropositivity rates and Geometric Mean Titres (GMT) (Total cohort)*

Antibody	Group	Timing	GMT	L.L.	U.L.	MIN	MAX
A/NEW-CALEDONIA	Fluarix™ IM	PRE	66.3	45.8	96.0	<10.0	905.0
		PI(D21)	725.0	536.2	980.2	80.0	5120.0
	Fluarix™ ID with IDD	PRE	34.3	24.1	48.8	<10.0	640.0
		PI(D21)	313.3	223.1	440.1	28.0	2560.0
A/PANAMA	Fluarix™ IM	PRE	40.6	28.2	58.3	<10.0	640.0
		PI(D21)	365.1	262.8	507.1	40.0	5120.0
	Fluarix™ ID with IDD	PRE	23.9	17.1	33.6	<10.0	453.0
		PI(D21)	220.2	149.0	325.3	10.0	5120.0
B/YAMANASHI	Fluarix™ IM	PRE	90.0	65.4	123.7	<10.0	640.0
		PI(D21)	983.6	741.0	1305.6	160.0	7241.0
	Fluarix™ ID with IDD	PRE	49.5	33.0	74.4	<10.0	1280.0
		PI(D21)	422.2	316.2	563.8	20.0	2560.0

PRE = pre-vaccination; PI (D21) = day 21 post vaccination

95% CI, L.L., and U.L. = 95% confidence intervals, lower and upper limit

- 15 The total number of subjects was 50 in each group

The differences for the three strains (New Caledonia, A/Panama, and B/Yamanashi), between the groups on day 0 were non-significant ( $p > 0.05$ ). On day 21, significant ( $p < 0.0001$ ) differences were observed between the ID group and the IM group.

However, when the increases in titres from day 0 to day 21 (conversion factor, see Table below) were compared, no significant difference was measured ( $p>0.05$ ) from one group to the other, meaning that the increases were globally comparable.

- 5 HI results do not allow discrimination between the intradermal vaccine group and the Fluarix™ intramuscular vaccine group.

*Conversion factor (Total cohort).*

Group	N	A/N-Caledonia [95 % CI]	A/Panama [95 % CI]	B/Yamanashi [95 % CI]
Fluarix IM.	50	10.6 [7.2 – 15.6]	9.3 [6.0 – 14.2]	10.9 [7.6 – 15.7]
Fluarix using ID delivery device	50	9.1 [6.2 – 13.3]	9.2 [5.6 – 15.2]	8.5 [5.7 – 12.8]

- 10 The conversion factor (fold increase in serum HI GMTs on day 21 compared to day 0) varies from 8.5 to 10.9 according to the virus strains and the route of administration (see Table above). This conversion factor is superior to the 2.5 fold increase in GMT required by the European Authorities.
- 15 An analysis of variance with the factor treatment as classification criterion was used to compare the conversion factors. No significant difference was measured between the treatment groups ( $p>0.05$ )

**Seroprotection rate**

- 20 The seroprotection rate shown in the Table below is defined as the percentage of vaccinees with a serum HI titre  $\geq 40$  after vaccination.

*Distribution of individual antibody titres and protection rates (Total cohort)*

Antibody	Group	Timing	N	<40		$\geq 40$	
				n	%	n	%

A/NEW- CALEDONIA	Fluarix™ IM	PRE	50	15	30.0	35	70.0
		PI(D21)	50	0	0.0	50	100.0
	Fluarix™ ID with IDD	PRE	50	26	52.0	24	48.0
		PI(D21)	50	1	2.0	49	98.0
A/PANAMA	Fluarix™ IM	PRE	50	21	42.0	29	58.0
		PI(D21)	50	0	0.0	50	100.0
	Fluarix™ ID with IDD	PRE	50	29	58.0	21	42.0
		PI(D21)	50	2	4.0	48	96.0
B/YAMANASHI	Fluarix™ IM	PRE	50	8	16.0	42	84.0
		PI(D21)	50	0	0.0	50	100.0
	Fluarix™ ID with IDD	PRE	50	20	40.0	30	60.0
		PI(D21)	50	1	2.0	49	98.0

PRE = pre-vaccination, PI (D21) = day 21 post vaccination

N = number of subjects tested.

n = number of subjects with HI titres < or ≥ 40

% =  $n/N \times 100\%$

5 < 40: Titres less than 40 HIU

≥ 40: Titres more than or equal to 40 HIU

At day 21, the seroprotection rates in the groups ranged from 96% to 100% for the different virus strains. In terms of protection, this means that more than 95% of the subjects (whatever the route of administration) had a serum HI titre ≥ 40 after vaccination and were deemed to be protected against the three strains. This rate is superior to the seroprotection rate of 70% required in the 18-60 year old population, by the European Authorities.

#### 15 Seroconversion rate.

The seroconversion factor given in the Table below is defined as the percentage of vaccinees that have at least a 4-fold increase in serum HI titres after vaccination.

*Vaccine responses and seroconversion (Total cohort)*

Antibody	Group	Prevacc. Status	N	Responders			
				n	%	95% CI LL	UL

A/NEW-CALEDONIA	Fluarix™ IM	Total	50	39	78	64	88.5
	Fluarix™ ID with IDD	Total	50	37	74	59.7	85.4
A/PANAMA	Fluarix™ IM	Total	50	36	72	57.5	83.8
	Fluarix™ ID with IDD	Total	50	33	66	51.2	78.8
B/YAMANASHI	Fluarix™ IM	Total	50	40	80	66.3	90
	Fluarix™ ID with IDD	Total	50	35	70	55.4	82.1

95% CI, L.L., and U.L. = 95% confidence intervals, lower and upper limit

N = number of subject tested

n = number of subject responding to vaccination.

% =  $n/N \times 100$  %

5

To be deemed effective and according to European Authority requirements, a vaccine should induce a seroconversion rate greater than 40% in the 18-60 year old population. In this study, the seroconversion rate was greater than 65% for the groups.

## 10 Reactogenicity

The intradermal administration of vaccine was safe (no serious adverse events were reported) and clinically well tolerated with very few reports of general symptoms related to vaccination.

## 15 Conclusions

- Fluarix™ induced good immune responses for each strain with a high seroconversion rate after one dose whatever the route of administration (ID or IM).
- There was no significant difference between the immune response elicited by 1/5 dose of Fluarix™ given intradermally and by the full dose administrated by the IM route.
- Both vaccinations fulfilled the requirement of the European Authorities for influenza inactivated vaccines in the 18-60 year old population, i.e.,

20

- Induce a seroconversion rate greater than 40%.
- Increase the geometric mean titre by more than 2.5.
- Elicit a seroprotection rate of 70% .

5

**Example 5: Immunogenicity and Reactogenicity of Flu ID: Study 2****Preparation of influenza virus antigen preparation**

- 10 Monovalent split vaccine was prepared according to the following procedure.

**Preparation of virus inoculum**

- On the day of inoculation of embryonated eggs a fresh inoculum is prepared by mixing the working seed lot with a phosphate buffered saline containing gentamycin sulphate at 0.5 mg/ml and hydrocortisone at 25 µg/ml. (virus strain-dependent). The virus inoculum is kept at 2-8°C.

**Inoculation of embryonated eggs**

- Nine to eleven day old embryonated eggs are used for virus replication. Shells are decontaminated. The eggs are inoculated with 0.2 ml of the virus inoculum. The inoculated eggs are incubated at the appropriate temperature (virus strain-dependent) for 48 to 96 hours. At the end of the incubation period, the embryos are killed by cooling and the eggs are stored for 12-60 hours at 2-8°C.

25 **Harvest**

The allantoic fluid from the chilled embryonated eggs is harvested. Usually, 8 to 10 ml of crude allantoic fluid is collected per egg.

**Concentration and purification of whole virus from allantoic fluid**

30 **1 Clarification**

The harvested allantoic fluid is clarified by moderate speed centrifugation (range: 4000 – 14000 g).

## 2 Adsorption step

To obtain a  $\text{CaHPO}_4$  gel in the clarified virus pool, 0.5 mol/L  $\text{Na}_2\text{HPO}_4$  and 0.5mol/L  $\text{CaCl}_2$  solutions are added to reach a final concentration of  $\text{CaHPO}_4$  of 1.5 g to 3.5 g  $\text{CaHPO}_4$ /litre depending on the virus strain.

5

After sedimentation for at last 8 hours, the supernatant is removed and the sediment containing the influenza virus is resolubilised by addition of a 0.26 mol/L EDTA- $\text{Na}_2$  solution, dependent on the amount of  $\text{CaHPO}_4$  used.

## 10 3 Filtration

The resuspended sediment is filtered on a 6 $\mu\text{m}$  filter membrane.

## 4 Sucrose gradient centrifugation

The influenza virus is concentrated by isopycnic centrifugation in a linear sucrose  
15 gradient (0.55 % (w/v)) containing 100  $\mu\text{g/ml}$  Thiomersal. The flow rate is 8 – 15 litres/hour.

At the end of the centrifugation, the content of the rotor is recovered by four different fractions (the sucrose is measured in a refractometer):

- |    |   |            |                              |
|----|---|------------|------------------------------|
| 20 | - | fraction 1 | 55-52% sucrose               |
|    | - | fraction 2 | approximately 52-38% sucrose |
|    | - | fraction 3 | 38-20% sucrose*              |
|    | - | fraction 4 | 20- 0% sucrose               |

\* virus strain-dependent: fraction 3 can be reduced to 15% sucrose.

25

For further vaccine preparation, only fractions 2 and 3 are used.

Fraction 3 is washed by diafiltration with phosphate buffer in order to reduce the sucrose content to approximately below 6%. The influenza virus present in this  
30 diluted fraction is pelleted to remove soluble contaminants.

The pellet is resuspended and thoroughly mixed to obtain a homogeneous suspension. Fraction 2 and the resuspended pellet of fraction 3 are pooled and phosphate buffer is

added to obtain a volume of approximately 40 litres, a volume appropriate for 120,000 eggs/batch. This product is the monovalent whole virus concentrate.

### **5 Sucrose gradient centrifugation with sodium deoxycholate**

- 5 The monovalent whole influenza virus concentrate is applied to a ENI-Mark II ultracentrifuge. The K3 rotor contains a linear sucrose gradient (0.55 % (w/v)) where a sodium deoxycholate gradient is additionally overlayed. Tween 80 is present during splitting up to 0.1 % (w/v) and Tocopherol succinate is added for B-strain- viruses up to 0.5 mM. The maximal sodium deoxycholate concentration is 0.7-1.5 % (w/v) and  
10 is strain dependent. The flow rate is 8 – 15 litres/hour.

- At the end of the centrifugation, the content of the rotor is recovered by three different fractions (the sucrose is measured in a refractometer) Fraction 2 is used for further processing. Sucrose content for fraction limits (47-18%) varies according to strains  
15 and is fixed after evaluation:

### **6 Sterile filtration**

- The split virus fraction is filtered on filter membranes ending with a 0.2 µm membrane. Phosphate buffer containing 0.025 % (w/v) Tween 80 and (for B strain  
20 viruses) 0.5 mM Tocopherol succinate is used for dilution. The final volume of the filtered fraction 2 is 5 times the original fraction volume.

### **7 Inactivation**

- The filtered monovalent material is incubated at  $22 \pm 2^{\circ}\text{C}$  for at most 84 hours  
25 (dependent on the virus strains, this incubation can be shortened). Phosphate buffer containing 0.025% (w/v). Tween 80 is then added in order to reduce the total protein content down to max. 250 µg/ml. For B strain viruses, a phosphate buffered saline containing 0.025% (w/v) Tween 80 and 0.25 mM Tocopherol succinate is applied for dilution to reduce the total protein content down to 250 µg/ml. Formaldehyde is  
30 added to a final concentration of 50 µg/ml and the inactivation takes place at  $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for at least 72 hours.

### **8 Ultrafiltration**

The inactivated split virus material is concentrated at least 2 fold in a ultrafiltration unit, equipped with cellulose acetate membranes with 20 kDa MWCO. The Material is subsequently washed with phosphate buffer containing 0.025 % (w/v) Tween 80 and following with phosphate buffered saline containing 0.01 % (w/v) Tween. For B strain virus a phosphate buffered saline containing 0.01% (w/v) Tween 80 and 0.1 mM Tocopherol succinate is used for washing.

#### 9 Final sterile filtration

The material after ultrafiltration is filtered on filter membranes ending with a 0.2 µm membrane. Filter membranes are rinsed and the material is diluted if necessary such that the protein concentration does not exceed 1,000 µg/ml but haemagglutinin concentration exceeds 180 µg/ml with phosphate buffered saline containing 0.01% (w/v) Tween 80 and (for B strain viruses) 0.1 mM Tocopherol succinate.

#### 10 Storage

The monovalent final bulk is stored at 2 – 8°C for a maximum of 18 months.

#### Example 6 – Preparation of influenza vaccine

Monovalent final bulks of three strains, A/New Caldonia/20/99 (H1N1) IVR-116, A/Panama/2007/99 (H3N2) Resvir-17 and B/Johannesburg/5/99 were produced according to the method described in Example 5.

#### 25 Pooling

The appropriate amount of monovalent final bulks were pooled to a final HA-concentration of 60 µg/ml for A/New Caldonia/20/99 (H1N1) IVR-116, A/Panama/2007/99 (H3N2) Resvir-17, respectively and of 68 µg/ml for B/Johannesburg/5/99. Tween 80, Triton X – 100 and Tocopherol succinate were adjusted to 1,000 µg/ml, 110 µg/ml and 160 µg/ml, respectively. The final volume was adjusted to 3 l with phosphate buffered saline. The trivalent pool was filtered



ending with 0.8 µm cellulose acetate membrane to obtain the trivalent final bulk.

Trivalent final bulk was filled into syringes at least 0.165 mL in each.

### Vaccine administration

- 5 The vaccine was supplied in pre-filled syringes and was administered intradermally in the deltoid region. The intradermal (ID) needle was as described in EP1092444, having a skin penetration limiter to ensure proper intradermal injection. Since formation of a wheal (papule) at the injection site demonstrates the good quality of ID administration, the investigator with the subject measured the exact size of the wheal
- 10 30 minutes after vaccination.

One dose (100 µl) contained the following components:

HEMAGGLUTININ FROM THREE INFLUENZA STRAINS			
	A/NEW CALEDONIA/20/99	:	6.0 µg
	A/PANAMA/2007/99	:	6.0 µg
	B/JOHANNESBURG 5/99	:	6.0 µg
THIOMERSAL PRESERVATIVE		:	0.4 µg – 0.8 µg

The above vaccine was compared a standard trivalent split influenza vaccine:

- Fluarix™. The Fluarix vaccine was supplied in pre-filled syringes and was
- 15 administered intramuscularly in the deltoid muscle. A needle of at least 2.5 cm / 1 inch in length (23 gauge) was used to ensure proper intramuscular injection.

One dose (0.5 ml) contained the following components:

HEMAGGLUTININ FROM THREE INFLUENZA STRAINS			
	A/NEW CALEDONIA/20/99	:	15.0 µg
	A/PANAMA/2007/99	:	15.0 µg
	B/JOHANNESBURG 5/99	:	15.0 µg
THIOMERSAL PRESERVATIVE		:	50.0 µg

20

### Results

The mean age of the total cohort at the time of vaccine administration was  $70.4 \pm 6.2$  years Standard Deviation (S.D.), the female/male ratio was 1.7:1.

Immunogenicity results: Analysis of derived immunogenicity variables was as follows:								
Variable		Flu-red ID (N = 65)			Fluarix™ IM (N = 65)			
GMT		GMT	LL	UL	GMT	LL	UL	
A/NEW CALEDONIA	PRE	99.5	76.9	128.7	90.0	70.1	115.7	
	POST	165.1	129.2	211.0	174.3	133.3	227.9	
A/PANAMA	PRE	75.5	54.7	104.2	69.2	51.9	92.4	
	POST	128.6	99.1	166.8	164.3	126.0	214.1	
B/JOHANNESBURG	PRE	236.0	187.7	296.8	222.6	176.9	280.2	
	POST	341.2	276.0	421.7	402.4	312.1	518.9	
Seroconversion rate		%	LL	UL	%	LL	UL	
A/NEW CALEDONIA		15.4	7.6	26.5	18.5	9.9	30.0	
A/PANAMA		20.0	11.1	31.8	29.2	18.6	41.8	
B/JOHANNESBURG		9.2	3.5	19.0	16.9	8.8	28.3	
Conversion factor		GMR	LL	UL	GMR	LL	UL	
A/NEW CALEDONIA		1.7	1.4	2.0	1.9	1.6	2.3	
A/PANAMA		1.7	1.4	2.1	2.4	1.9	3.0	
B/JOHANNESBURG		1.4	1.2	1.7	1.8	1.5	2.1	
Seroprotection rate		%	LL	UL	%	LL	UL	
A/NEW CALEDONIA	PRE	87.7	77.2	94.5	90.8	81.0	96.5	
	POST	92.3	83.0	97.5	96.9	89.3	99.6	
A/PANAMA	PRE	75.4	63.1	85.2	81.5	70.0	90.1	
	POST	90.8	81.0	96.5	93.8	85.0	98.3	
B/JOHANNESBURG	PRE	98.5	91.7	100.0	96.9	89.3	99.6	
	POST	100.0	94.5	100.0	98.5	91.7	100.0	
N: number of subjects with available results; %: percentage of subjects within the given parameter, LL/UL: lower and upper limit of 95% CI; Pre: at the time of vaccine administration; Post: 21 days after the vaccine dose								

- 5 Injection site pain, reported by 10/65 (15.4%) vaccinees, was the most common symptom following IM administration of Fluarix™. In the ID group, pain was reported by 3/65 (4.6%) vaccinees. This difference was statistically significant ( $p=0.038$ ; Fisher exact test). Frequency of pain is therefore reduced when using ID administration.

## Conclusions

ID administration of a flu vaccine provides equivalent (100%) seroprotection in an elderly population.

A comparable response to vaccination in terms of geometric mean titers,  
5 seroprotection rates, seroconversion rates and conversion factors was found in IM and ID vaccinated individuals where the ID group received 2.5-fold less antigen.

There was no discernible difference in the overall incidence of vaccine-related solicited/unsolicited systemic symptoms in the two treatment groups.

10

### Example 7 – Intradermal delivery using standard needle

Immunogenicity of the split influenza vaccine was assessed by ID delivery in pigs  
15 using a standard needle.

Pigs show important physiologic similarities to humans, and pig skin in particular is quite similar to human skin in terms of appearance, anatomy, and physiology.

Therefore, studies where properties of the skin are important may be assessed in the most relevant manner in pigs. The pig also has the advantage that it is a natural host  
20 for influenza infection (A strains only) and thus testing of vaccine candidates in pigs is relevant.

In a first immunogenicity study conducted in 4 week old pigs, 3 groups of 6 pigs each were primed by intranasal administration of whole, inactivated, trivalent influenza (50 µg each HA adjuvanted with 0.5% Laureth 9) in a total volume of 200 µl – 100 µl  
25 administered in each nostril using a Pfeiffer intranasal device (described for example in WO 91/13281, EP 311 863 B and EP 516 636 B, commercially available from Pfeiffer GmbH). A second priming dose was administered at day 11.

On day 39 the animals were vaccinated by either the ID (Fluarix™ or PBS control) or  
30 IM (Fluarix™ only) route. Animals receiving IM vaccination were immunized with trivalent Fluarix™ (15 µg each HA of strains A/New Caledonia H1N1, A/Panama

H3N2, and B/Johannesburg) in 0.5 ml administered in the front leg. Animals receiving ID vaccination were immunized with trivalent Fluarix™ (3 µg each HA) or PBS in 0.1 ml administered using a standard needle.

5 Blood samples were obtained on day 53 and tested for anti-influenza activity using ELISA assays.

The results of this first immunogenicity study are presented in Fig 1 which shows the results obtained from this study using the strain-specific ELISA readout.

**Legend to Figure 1:**

- 10 Group 1: 2 IN Primings (Trivalent 50 µg); trivalent vaccine IM 15 µg HA  
Group 2: 2 IN Primings (Trivalent 50 µg); trivalent vaccine ID 3 µg HA  
Group 3: 2 IN Primings (Trivalent 50 µg); PBS ID

The results confirm the immunogenicity of the trivalent influenza vaccine administered to primed pigs by either the IM or ID route.

15

**Example 8 – Intradermal delivery of adjuvanted influenza vaccine**

**Protocol**

20 Guinea pigs were primed on Day 0 with 5 µg trivalent whole inactivated Flu virus in 200 µl, intranasally.

Vaccination - Day 28 - Vaccine containing 0.1 µg HA each per strain trivalent split Flu vaccine prepared as described in Examples 5 and 6 except that the pooling (Example 6) resulted in a final concentration for each antigen of 1.0 µg/ml to give a dose of 0.1 µg in 100 µl compared to 60 µg/ml in Example 6. The final trivalent  
25 formulation was administered intradermally using tuberculin syringes, either adjuvanted or unadjuvanted, in 100 µl.

Bleeding - Day 42.

The effect of adjuvantation was assessed by measuring antibody responses by HI assay (day 0, 28, 42).

All ID experiments were carried out using a standard needle.

### Results

5

G1-G5 refer to 5 groups of guinea pigs, 5 per group.

- G1 Split trivalent thiomersal reduced 0.1µg  
 G2 Split trivalent thio red 0.1µg + 3D-MPL 50µg  
 10 G3 Split trivalent thio red 0.1µg + 3D-MPL 10µg  
 G4 Split trivalent thio red 0.1µg + 3D-MPLin 50µg + QS21 50µg  
 G5 Split trivalent thio red 0.1µg + 3D-MPLin 10µg + QS21 10µg

Note 3D-MPLin + QS21 refers to an adjuvant formulation which comprises a  
 15 unilamellar vesicle comprising cholesterol, having a lipid bilayer comprising dioleoyl  
 phosphatidyl choline, wherein the QS21 and the 3D-MPL are associated with, or  
 embedded within, the lipid bilayer. Such adjuvant formulations are described in EP 0  
 822 831 B, the disclosure of which is incorporated herein by reference.

20 HI titres anti-A/New Caledonia/20/99

NC	Pre-immun	Pre-boost	Post-boost
G1	5	10	92
G2	5	10	70
G3	5	11	121
G4	7	9	368
G5	5	10	243

25 HI titres anti-A/Panama/2007/99

P	Pre-immun	Pre-boost	Post-boost
G1	5	485	7760
G2	5	279	7760
G3	5	485	8914
G4	7	485	47051
G5	5	320	17829

HI titres anti-B/Johannesburg/5/99

J	Pre-immun	Pre-boost	Post-boost
G1	5	23	184
G2	5	11	121
G3	5	11	70

G4	6	15	557
G5	5	13	320

The data presented in this example confirm and extend the results obtained in the previous example, conducted in pigs. ID administration of trivalent Flu vaccine induces a strong immune response in primed animals (guinea pigs in addition to pigs). In addition, the potential for adjuvants to further boost this immune response is exemplified. Two different doses of 3D-MPLin + QS21 were shown to significantly boost the antibody titres induced by vaccination with unadjuvanted split trivalent Flu antigen. Thus, a Flu ID vaccine can be successfully adjuvanted and the resulting product can induce enhanced immune responses in vaccinated individuals.

## CLAIMS

1. The use of an influenza antigen preparation obtainable by the following  
5 process, in the manufacture of an intradermal flu vaccine:
- (i) harvesting of virus-containing material from a culture;
  - (ii) clarification of the harvested material to remove non-virus material;
  - (iii) concentration of the harvested virus;
  - (iv) a further step to separate whole virus from non-virus material;
  - 10 (v) splitting of the whole virus using a suitable splitting agent in a density  
gradient centrifugation step;
  - (vi) filtration to remove undesired materials;
- wherein the steps are performed in that order but not necessarily consecutively.
- 15 2. The use according to claim 1 wherein the intradermal flu vaccine is a trivalent vaccine.
- 3 The use according to claim 1 or claim 2 wherein the virus is grown on  
embryonated hen eggs and the harvested material is allantoic fluid.
- 20 4. The use according to any one of claims 1 to 3 wherein the clarification step is  
performed by centrifugation at a moderate speed.
5. The use according to any one of claims 1 to 4 wherein the concentration step  
25 employs an adsorption method such as  $\text{CaHPO}_4$  adsorption.
6. The use according to any one of claims 1 to 5 wherein the further separation  
step (iv) is a zonal centrifugation separation using a sucrose gradient.
- 30 7. The use according to claim 6 wherein the splitting step is performed in a  
further sucrose gradient, wherein the sucrose gradient contains the splitting agent.

8. The use according to claim 7 wherein the splitting agent is sodium deoxycholate.
9. The use according to any one of claims 1 to 8 wherein the filtration step (vi) is  
5 an ultrafiltration step which concentrates the split virus material.
10. The use according to any one of claims 1 to 9 wherein there is at least one sterile filtration step, optionally at the end of the process.
- 10 11. The use according to any one of claims 1 to 10 wherein an inactivation step is performed prior to the final filtration step.
12. The use according to any one of claim 1 to 11 wherein the method comprises the further step of adjusting the concentration of one or more detergents in the vaccine  
15 composition.
13. The use according to any one of claims 1 to 12 wherein the vaccine is provided in a dose volume of between about 0.1 and about 0.2 ml.
- 20 14. The use according to any one of claims 1 to 13 wherein the vaccine is provided with an antigen dose of 1-7.5  $\mu\text{g}$  haemagglutinin per strain of influenza present.
15. The use according to any one of claims 1 to 14 wherein the vaccine further  
25 comprises an adjuvant such as an adjuvant comprising a combination of cholesterol, a saponin and an LPS derivative.
16. The use of a trivalent, split influenza antigen preparation in the manufacture of a vaccine for intradermal delivery.
- 30 17. The use according to claim 16 wherein the intradermal vaccine comprises at least one non-ionic surfactant.

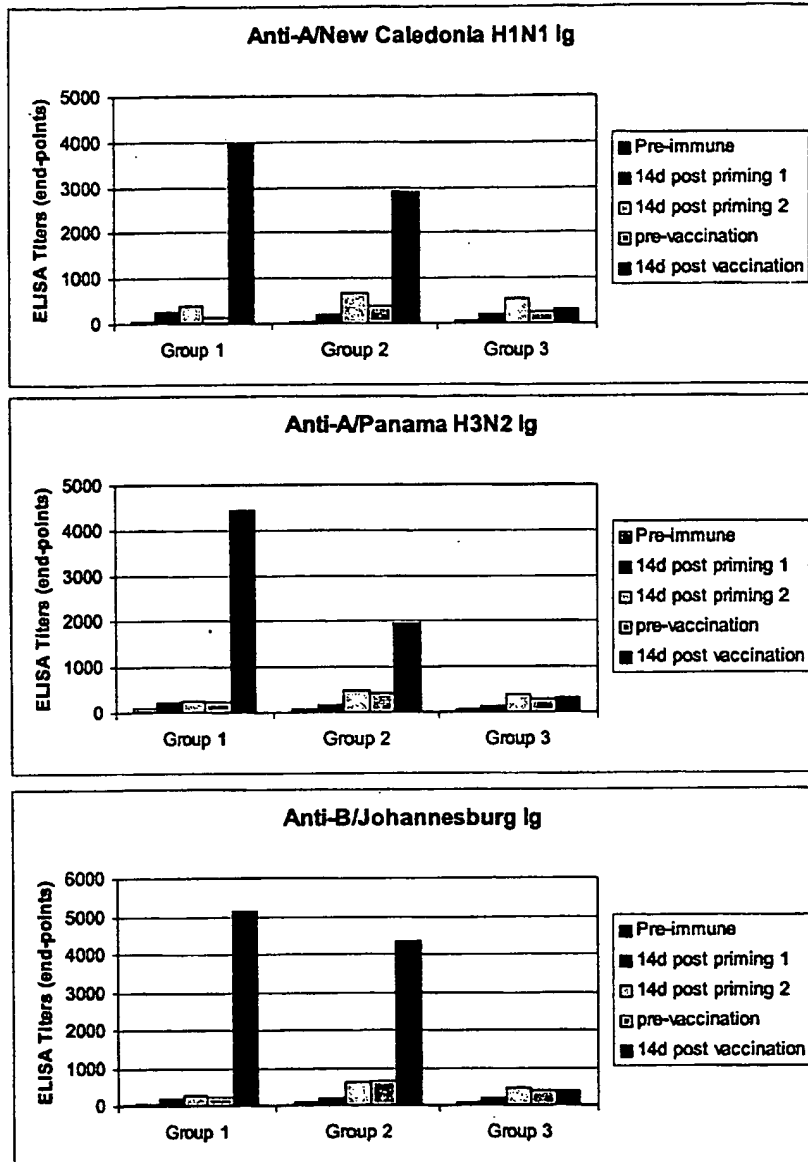


18. A pharmaceutical kit comprising an intradermal delivery device and an influenza vaccine obtainable by the following process:

- (i) harvesting of virus-containing material from a culture;
- (ii) clarification of the harvested material to remove non-virus material;
- 5 (iii) concentration of the harvested virus;
- (iv) a further step to separate whole virus from non-virus material;
- (v) splitting of the whole virus using a suitable splitting agent in a density gradient centrifugation step;
- (vi) filtration to remove undesired materials;
- 10 wherein the steps are performed in that order but not necessarily consecutively.

19. The pharmaceutical kit according to claim 18 wherein the intradermal delivery device is a short needle delivery device.

**Figure 1: Anti-Influenza Immunoglobulin Titers in Primed Pigs Following IM or ID Vaccination with Fluarix<sup>TM</sup>**



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 02/01843

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 A61K39/145 C12N7/04

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 19013 A (SMITHKLINE BEECHAM CORP ;SMITHKLINE BEECHAM BIOLOG (BE); DILLON SU) 1 September 1994 (1994-09-01)	1-11,16, 17
Y	page 27; claim 5; examples 25,26,30	13-15, 18,19
Y	--- VASIL'EVA R I ET AL: "STUDY OF THE RESULTS OF USING NEW INACTIVATED WHOLE-VIRION INFLUENZA VACCINES IN THE USSR" ZHURNAL MIKROBIOLOGII EPIDEMIOLOGII I IMMUNOBIOLOGII, no. 3, 1987, pages 38-42, XP008005763 ISSN: 0372-9311 page 42, column 2, paragraph 3; tables 1,2 --- -/--	1,13,14, 16,18,19



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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Date of the actual completion of the international search

16 July 2002

Date of mailing of the international search report

26/07/2002

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## INTERNATIONAL SEARCH REPORT

International Application No

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